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Phosphate transport: *from microperfusion to molecular cloning*

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Inorganic phosphate (P_i) is a constituent of important biological molecules (e.g. nucleic acids and phospholipids) and is essential for cellular energetics and signalling, protein synthesis as well as skeletal development in all mammalian organisms. Inadequate P_i supply causes bone malformations such as rickets and spinal deformations, whereas an excess in P_i is linked to vascular calcification or ectopic CaP_i deposits. In general whole body P_i homeostasis is maintained by trans-epithelial transport mechanisms in the small intestine and kidney where P_i is absorbed from the diet and reabsorbed from the glomerular filtrate, respectively. The renal proximal tubule is the main locus of P_i regulation so that under "steady-state" physiological conditions, renal P_i-excretion corresponds approximately to dietary P_i intake.

Membrane transport proteins belonging to the SLC34 solute carrier family[i] lie at the heart of maintaining P homeostasis$_i$. In the small intestine NaPi-IIb (SLC34A2) mediates luminal P_i uptake together with a paracellular component, whereas the renal isoforms NaPi-IIa (SLC34A1) and NaPi-IIc (SLC34A3) are responsible for P_i reabsorption in the proximal tubule. This Special Issue focussing on phosphate transport mediated by SLC34 proteins was conceived to coincide with an important milestone in renal physiology: the expression cloning of the first member of the SLC34 family (NaPi-IIa) just over a quarter of a century ago [35]. This, together with the subsequent identification of the other two SLC34 isoforms [24,43], has paved the way for a deeper understanding of the molecular basis of P_i homeostasis, many aspects of which are the subject of dedicated reviews in this issue. Whereas the pivotal role of SLC34 co transporters in maintaining P_i homeostasis is undisputed, other carriers such as PIT-1 and PIT-2 (SLC20 family) may also contribute to epithelial transport of P_i in intestine and the kidney. However, their respective contributions to overall P_i balance remain to be clarified and will not be the main focus of the present Special Issue.

Following the cloning of NaPi-IIa [35], the phosphate physiology field has progressed rapidly, benefitting from advances in molecular and cell biology, imaging and biophysical assays, as exemplified by the invited articles. Therefore it is easy to overlook how key aspects of our present knowledge of P_i handling had already been established prior to 1993. A brief reflection on the main findings of these earlier studies seems appropriate to set the scene for the developments that have taken place over the past 25 years.

Indeed, in the three decades that preceded the NaPi-IIa cloning, ground-breaking studies using *in vivo* preparations revealed fundamental properties of P_i transport physiology that are still valid today, including the location of active sites of absorption and reabsorption, the energetic basis of the transport mechanism and the identification of some of the key physiological regulators. One of the first attempts to identify the site of renal P_i reabsorption involved micropuncture of intact kidneys to compare samples of fluid from the renal tubules in normal and P_i-loaded rats [46]. It was shown that the reabsorption of filtered P_i occurs primarily in the proximal tubules and furthermore, it was concluded that this process probably involves an active transport process [46]. Later, using the standing droplet method [51] and simultaneous microperfusion of the peritubular capillaries performed *in situ* on superficial renal tubules (Fig 1A), Ullrich and colleagues at the MPI in Frankfurt established unequivocally that P_i is reabsorbed from the primary urine along the proximal tubule with highest rates occurring in the early part of the tubule [2]. These *in vivo* epithelial transport assays demonstrated that reabsorption of P_i in the proximal tubules is strictly dependent on the presence of sodium ions in the luminal medium (Fig. 1B). Importantly, the Frankfurt group provided the first insights into the regulation of renal P_i reabsorption. Their experiments

revealed that parathyroid hormone (PTH) down-regulates the rate of P_i reabsorption and that the reabsorption rate is influenced by dietary P_i [52,53] (Fig.1C). From such *in vivo* studies it became clear that proximal tubular reabsorption of P_i was indeed *not* paracellular, but rather involved a first transport step through the apical (brush-border) membrane of the epithelia and a subsequent exit step across the basolateral membrane [52,39]. In the following years the interest focused on functional aspects of the transport mechanism at the cell membranes facing either the tubular lumen or the peritubular interstitium. A novel technique, free flow electrophoresis, allowed the isolation of brush- border and basolateral membrane vesicles simultaneously [21,25] and enabled a direct comparison of the two transport steps. However, the isolated basolateral membranes (BLMV) were of random orientation and represented a mix of ”inside out” and ”outside out” vesicles, thus making the basolateral P_i exit step challenging to investigate. The findings obtained with free-flow separated vesicles were complemented by transport studies with basolateral membrane vesicles isolated by Percoll gradient. These seminal experiments established that the Na^+ dependent step is localized at the brush border membrane and the basolateral translocation of P_i is Na^+ independent [19]. Thus, proximal tubular reabsorption of P_i could be described as a classical secondary active transport process that relies on the Na/K-ATPase to establish the necessary electrochemical driving force. To conceptualise these findings, a model was proposed whereby the transmembrane Na^+ gradient drives the reabsorption of P_i via a Na^+-coupled P_i cotransporter system (Fig 3). A Na^+-independent mechanism (possibly an anion-exchanger) would then complete the net transcellular P_i movement from the primary urine to the peritubular space. Whereas the identity of the apical transport protein was soon to be established in Zurich, the membrane proteins mediating the basolateral step are still poorly understood, although the recent emergence of the retrovirus receptor (XPR1) as a candidate for this role appears most promising [16,1].

The pre-cloning P_i transport studies were advanced significantly by means of a modified procedure to isolate the proximal tubular brush border membrane vesicles (BBMVs). This was adapted from a protocol developed to isolate intestinal BBMV [26] and yielded pure and abundant BBMV and involved only a few differential centrifugation steps [6]. The isolated (renal) BBMV were of “right side out” orientation [18] and allowed the study of the transport characteristics in considerable detail in terms of kinetics and regulation. The first studies of P_i transport performed in the Ullrich laboratory (MPI, Frankfurt) [5] and later by the Saktor laboratory (NIH, Baltimore) [42] using renal proximal tubular BBMV established kinetic hallmarks of the transporter that would later serve as reference values for the cloned transporters. These laboratories confirmed the strict Na^+-dependence of transport and measured an apparent half-maximum concentration (K_m) of approximately 0.1 mM [25,42] (Fig.2). Furthermore, it was confirmed that P_i influx into BBMV was pH dependent, however there was no indication of a net movement of charge (i.e. electrogenic transport) [25,42]. Indeed, by having an excess of positive charge (i.e. 3 Na^+ to 1 divalent P_i) as would be eventually shown experimentally [8,14], the cotransport rate mediated by NaPi-IIa and NaPi-IIb would be enhanced by the inside negative membrane potential to provide an additional driving force. Meanwhile, these BBMV studies also allowed the investigation of various inhibitors of Na/P_i-cotransport, such as phosphonoformic acid [47,48]. In addition, dietary or hormonal interventions prior to BBMV isolation confirmed the inhibitory effect of PTH and the influence of dietary P_i as observed in microperfusion studies [38,45] (Fig. 2).

Importantly, the inhibitory/stimulatory effects were conserved in isolated membrane vesicles, i.e. independent of the cellular/organ context, and were manifest as changes in maximal transport rate ($\underline{V}_{max}$) but not K_m [33]. These observations underpinned the fundamental property of BBMV P_i transport, namely that the rate of P_i reabsorption was most likely proportional to the number of active transporters and not dependent on functional changes in the transporter itself.

In addition to the kidney, absorption of P_i along the small intestine also contributes to whole body P_i homeostasis [37]. Studies using isolated small intestinal membrane vesicles provided evidence for a Na^+-dependent and a Na^+-independent pathway across the brush border and basolateral membranes of enterocytes [40,10]. Interestingly, the Na^+-dependent component showed a divergent pH dependence compared to the renal transporter with significant transport activity at pH <7. Thus, for both, proximal tubules and small intestine, it was assumed that a single renal and intestinal Na^+-dependent transport system at the brush border membrane was being observed. Fig 3 depicts a schematic of the hypothesized single pathway of P_i through the apical membrane of proximal tubules. Based on the experimental evidence at the time, this model proposed a 2 Na^+:1 P_i stoichiometry and an allosteric regulation by protons that compete with the interaction of the Na^+ ions [17]. As divalent P_i is the prevalent form at physiological pH, it was also assumed that the transport process was electroneutral, contradicting previous studies with intact renal tubules in which electrogenicity was observed [15]. This model has since undergone several revisions as detailed in [13].

The molecular identity of the proteins involved in P_i transport was a pressing question and attempts were undertaken to identify the transporter by biochemical means in the precloning era. For example, given that PTH affects renal P_i-reabsorption and Na^+/P_i-cotransport in BBMVs in a cAMP-dependent manner, phosphorylation studies were performed with isolated BBMVs. These studies demonstrated cAMP-dependent changes of BBMV protein-phosphorylation, but the identity of those phospho-proteins remained unknown [20].

The cloning of NaPi-IIa [34,35] heralded a new era in the field by enabling the study of the transport mechanisms/characteristics in isolation after injection of cRNA into oocytes of *Xenopus laevis* [13]. By knowing the amino acid sequence, specific antibodies could be raised to enable specific *in situ* protein detection of each isoform. Studies using these antibodies allowed the detailed characterization of the cellular mechanisms that are involved in the physiological regulation by an increasing number of hormones. It was also possible to answer questions regarding the interaction with other proteins that are involved in the cellular regulation of Na^+/P_i-cotransport.

The collection of invited reviews in this Special Issue gives a comprehensive summary of the impressive progress that has been made in the P_i field since the early cloning days. The reviews have been grouped according to 5 themes: *Molecular and Mechanistic Aspects*, including interacting proteins[12,13,23,32,44]; *Physiological Regulation* [28,27,49]; *Human Mutations/Clinical Aspects and Diseases* [31,4]; *Non-renal Roles for SLC34 Proteins* [36,3] and, *Comparative Aspects* [41,54].

It is our hope that these articles will provide a useful overview for experts and non-experts alike and stimulate continued research in the P_i field. Indeed, it is notable that over the last 20 years, phosphate load in the Western diet has doubled due to phosphate containing food additives [50]. The associated spikes in post-brandial P_i levels are potentially related to

poor cardiovascular health and premature ageing, making excessive dietary P_i a public health concern [11]. Thus, research that furthers our understanding of SLC34 proteins at the molecular and systemic level is as timely as ever.

Figure legends

Figure 1: Evidence for transepithelial P_i transport in renal tissue demonstrated by microperfusion assays on the intact kidney.
A) General features of the experimental setup to perform microperfusion of superficial renal proximal tubules. Photograph shows the rat preparation: A: animal table; B: rat; C: inner kidney cup; D: outer kidney cup; E: outer cup holder; F: perfusion tube; G: suction; H: catheter in ureter; K: oil-filled micropipette (adapted and modified from [51]). *Inset* shows principle of split oil drop technique to measure net absorption from a single tubule: here, a double barrelled pipette is used to first inject oil into the tubule. The oil droplet is split by injection of the test solution (so-called standing droplet method). As the test solution is absorbed the oil droplets move towards one another. See [51] for details.
B) Transport of P_i is Na^+-dependent determined at two positions along the proximal tubule: in the presence of Na^+ on either side of the epithelia, achieved by means of double microperfusion involving continuous perfusion of peritubular capillaries and stop flow microperfusion of the proximal tubule [2]. The intraluminal P_i concentration (expressed as a % of the initial 2 mM concentration after 45 s exposure) is significantly reduced, whereas in the absence of Na^+ there is a small increase in P_i. Data taken from [2].
C) Effect of parathyroid hormone (PTH) on P_i transport in the early and late rat proximal tubule using the double microperfusion method as in B). The luminal perfusate contained initially 2mM P_i, hence a concentration difference (Δc) of maximally 2 mM could be detected indicating that all P_i is reabsorbed. When PTH was given intravenously this led to ~40% decrease of P_i transport in both early and late tubules within minutes (maximum at 15 minutes). Data taken from [53].

Figure 2: P_i transport assays performed with isolated proximal tubular brush border membrane vesicles (BBMVs).
A) Freeze fracture micrograph of BBMVs prepared from rat renal BBMVs. See [6] for details and methodology.
B) Effect of ion gradients on P_i transport. If the intravesicular space is Na^+-free, $^{32}P_i$ uptake initially overshoots and falls to an equilibrium value as the vesicles become loaded with Na^+ (Na^+ unloaded). No overshoot is observed with vesicles preloaded with Na^+ (Na^+ preloaded). With a K^+ gradient uptake is significantly reduced and not affected by preloading (open vs filled triangles). For more details, see original reference [25].
C) Time dependence of $^{32}P_i$ uptake into BBMVs demonstrating the effect of dietary P_i on BBMV activity. As in B) the uptake peaks and decreases as the vesicles become loaded with substrate, and the uptake eventually reaches an equilibrium exchange condition identical to the mannitol control. For vesicles prepared from animals on a low P_i diet, the peak uptake is significantly enhanced, consistent with having greater number of carriers expressed, as would be later shown by immunostaining of proximal tubules and protein analysis (e.g.[39]). Data modified from [45].

Figure 3. Pre-cloning model of the molecular mechanism from 1986 of renal proximal tubular Na-dependent P_i-reabsorption that accounts for apical entry of P_i [17]. At the apical

membrane the interaction of 2 Na^+ ions from the external medium allows divalent P_i to bind and be translocated by means of a secondary-active carrier. Protons compete with Na^+ ions to account for the pH dependence of transport. Studies on the cloned transporter have led to the revision of this model (see [13]). Basolateral exit of P_i was proposed to be mediated by Na^+-independent anion carrier (yellow) and the Na/K-ATPase maintains the driving force (purple).

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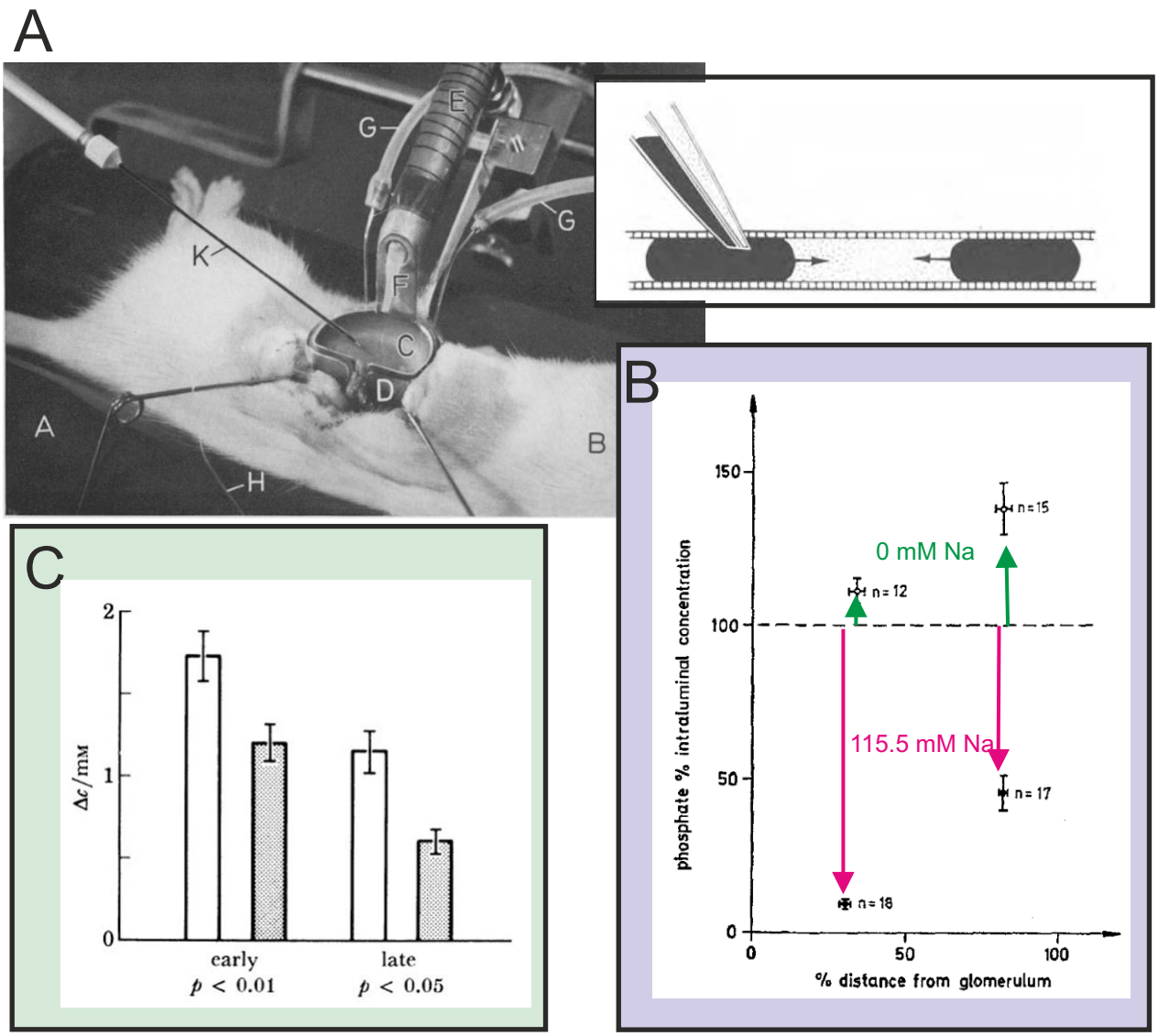
[i] Historically, the nomenclature of the cloned, mammalian P_i transporters followed a strictly chronological convention, beginning with the type 1 (NaPi-I) [55,9] (since shown to be related to an anion conductance and not directly mediate Na-dependent P_i transport [7]); the type II (NaPi-II [35] or npt2) and the type III (NaPi-III [29,30], or Pit-1,2).The widely used the solute carrier nomenclature [22] assigns the type I transporters to the SLC17 gene family; type II Na-P_i transporters to SLC34 and type III transporters to SLC20. respectively. The SLC system is increasingly used, i.e. SLC34A1, SLC34A2 and SLC34A3 for the human type II transporters NaPi-IIa, NaPi-IIb and NaPi-IIc, respectively. The structurally similar isoforms are grouped into the same sub-family (SLC34A) with individual indices (1,2,3). This nomenclature is easily applicable to most vertebrates, it only becomes convoluted in the case of fish that have undergone genome duplications followed by lineage specific gene losses.

Figure 1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n = 12
115.5 mM Na
n = 17
n = 18
phosphate % intraluminal concentration
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50
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% distance from glomerulum
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2
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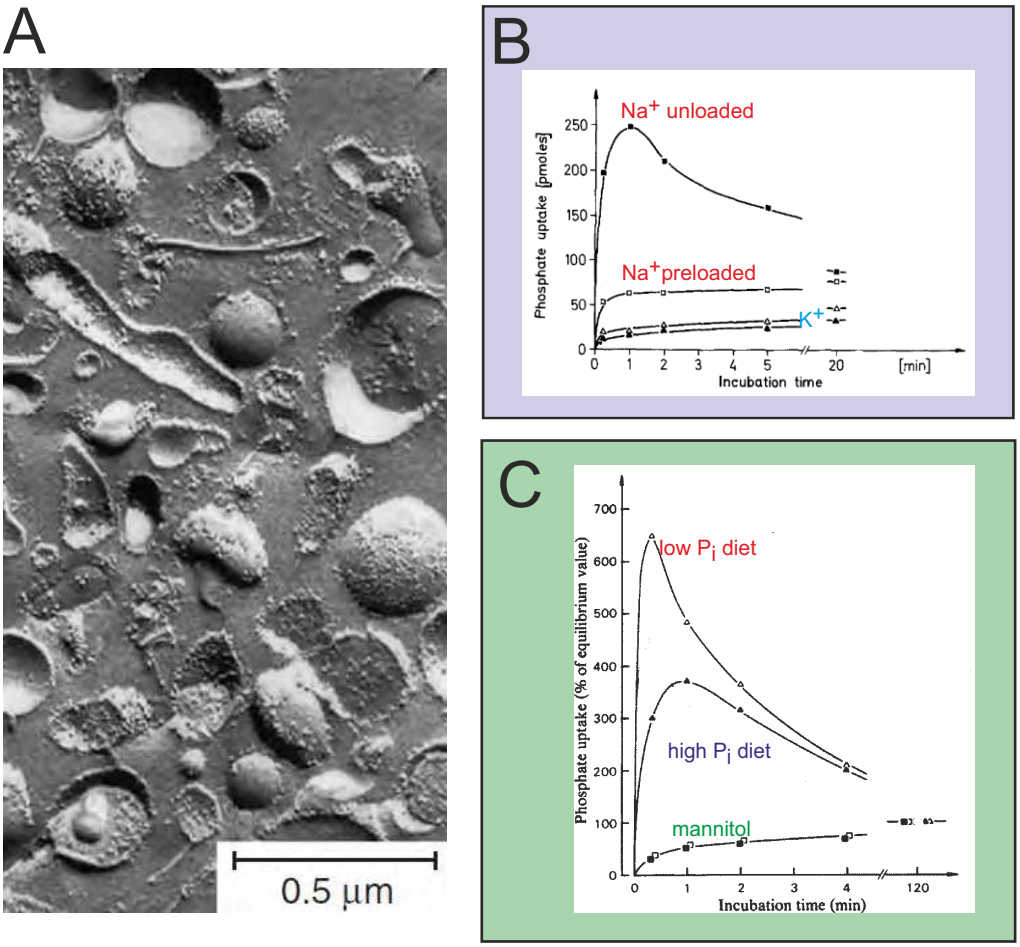
Δc/mM
early
p < 0.01
late
p < 0.05

Figure 2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B
Na+ unloaded
Na+preloaded
K+
Phosphate uptake [pmoles]
250
200
150
100
50
0
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Incubation time
C
low Pi diet
high Pi diet
mannitol
Phosphate uptake (% of equilibrium value)
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600
500
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300
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100
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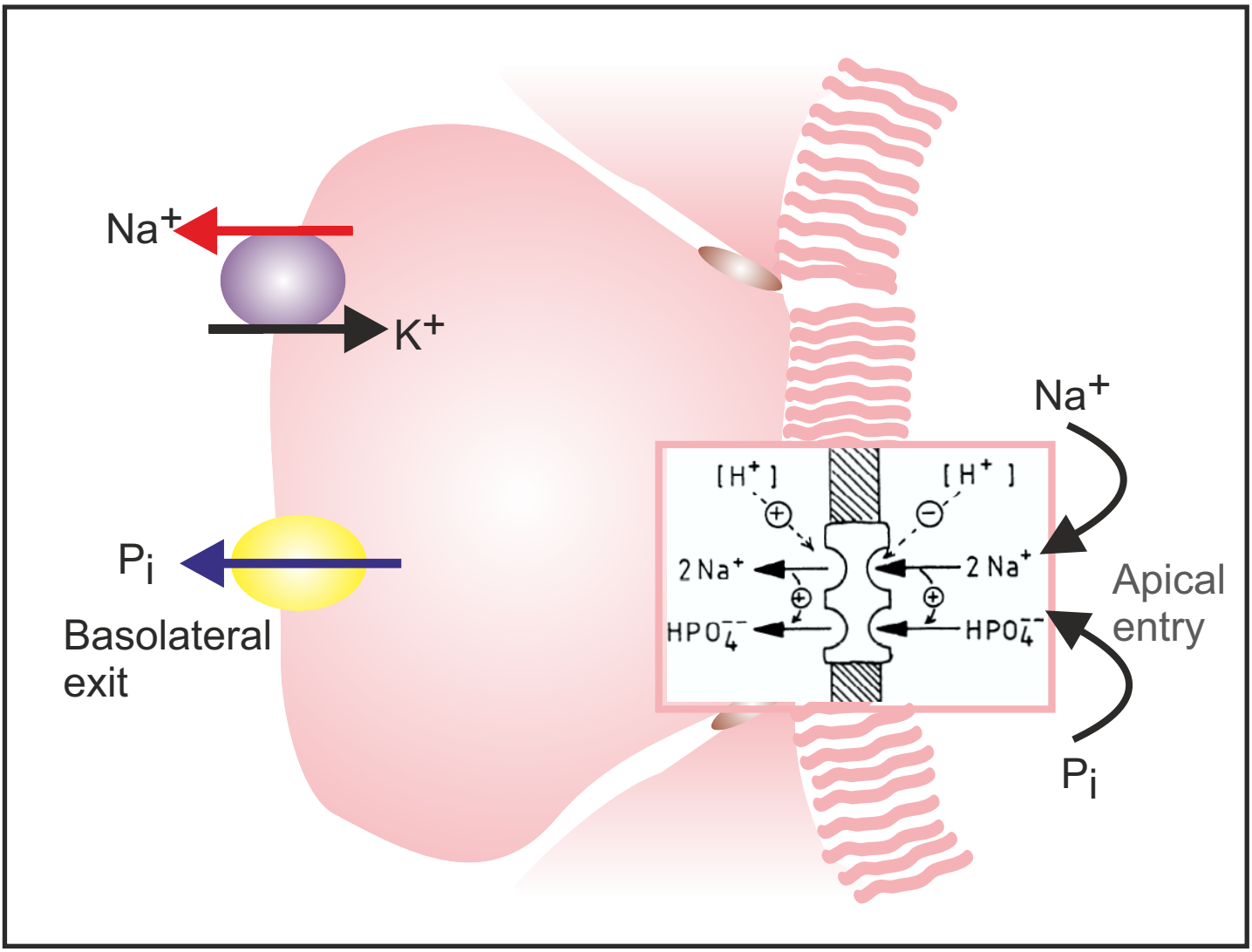
Incubation time (min)

Figure 3


Na+
K+
Pi
Basolateral exit
Na+
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2 Na+
2 Na+
HPO4--
HPO4--
Apical entry
Pi